17.8.2015

MONDAY, 8/17

Petra

Over-extension PCR for AtoB and CenA:
Since Gibson assembly and ELIC for AtoB and CenA haven't worked, we will test over-extension PCR for them.

Made mini-gradients (3 tubes/ reaction) for Atob And CenA constructs to test different annealing temperatures:

4x reaction mix for AtoB (100 ul)
69,77 µl H2O (Calculate the amount of water based on how much DNA you use)
20 µl 5 x Buffer
3 µl 10mM dNTP mix
2 µl KAPA HiFi HotStart DNA Polymerase
1,22 ul AtoB Part 1
0,71 ul Fixed AtoB Part 2
0,62 ul AtoB part 3
2,68 ul AtoB part 4

Pipetted 25 ul Reaction mix to 3 PCR tubes. Marked the tubes: A1 = AtoB reaction 1 ... A3 = AtoB reaction 3. Pipetted the remaining reaction mix (~20 ul) to 4th tube because wanted to try PCR with it.

Gradient:
65 °*C:* A1
66,4 °C: A2
67,2 °C: A3
68,1 °C: A4 (the sample with 20 ul reaction mix)

PCR Program (AtoB):
95° *C - 3 min*
98° *C - 30 sec*
65-68,1 °*C - 30 sec*
72° C - 4,5 min
72° *C - 10 min*
4° *C - forever*
Repeat the underlined steps 15 times

4x reaction mix for CenA (100 ul)
71,64 µl H2O (Calculate the amount of water based on how much DNA you use)
20 µl 5 x Buffer
3 µl 10mM dNTP mix
2 µl KAPA HiFi HotStart DNA Polymerase
1,54 ul CenA Part 1
1,12 ul CenA Part 2
0,7 ul Bglx (from 2nd PCR)

Pipetted 25 ul Reaction mix to 3 PCR tubes. Marked the tubes: C1 = CenA reaction 1 ... C3 = CenA reaction 3. Pipetted the remaining reaction mix (~20 ul) to 4th tube because wanted to try PCR with it.

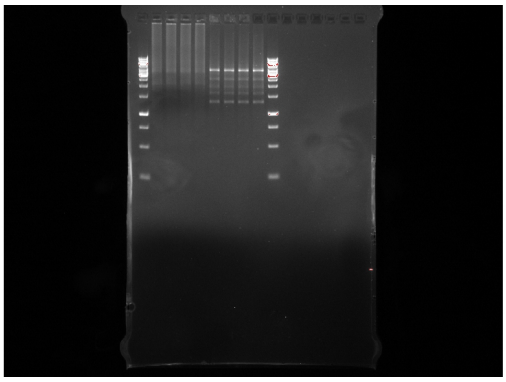
Gradient:
72 °C: C1
71,3 °C: C2
69,7 °C: C3
68,1 °C: C4 (the sample with 20 ul reaction mix)

PCR Program (CenA):
95° *C - 3 min*
98° *C - 30 sec*
68,1-72 °*C - 30 sec*

72° C - 4,5 min
72° C - 10 min
4° C - forever
Repeat the underlined steps 15 times

Made a 1,3 % agarose gel with ETBR. Ran 5 ul samples of each PCR reaction with 1 ul LD (8 samples in total). Pipetting order:
1.Ladder
2. AtoB 1
3. AtoB 2
4. AtoB 3
5. AtoB 4
6. CenA 4
7. CenA 3
8. CenA 2
9. CenA 1
10. Ladder

Geldoc_2015-08-17_15hr_43min_AtoB_CAR_oe-PCR.jpg



According to the gel pic oe-PCR for CenA was succesful.

Transforming CAR construct to the biobrick backbone:
Four CAR constructs were sent to sequencing, but since we don't have the results yet we decided to work with all of them: CAR Gibson 2, CAR Gibson 3, CAR Gibson 4 and CAR Gibson 7.
Used NeBuffer 3.1 for restriction with EcoRI & Pst1.
Restricted the constructs (CAR Gibson 2, CAR Gibson 3, CAR Gibson 4 and CAR Gibson 7) and linearized pSB1C3 backbone (AH009) with EcoRI and PstI according to the protocol. Amounts of DNA and water used are listed in Table 1.

Table1

Construct	DNA pipetted for 250 ng (ul)	Water added (ul)
CAR Gibson 2	2,56	18,74
CAR Gibson 3	2,24	19,00
CAR Gibson 4	2,1	19,2
CAR Gibson 7	2,66	18,60
AH009 = pSB1C3 (linearized)	10,0	11,3

Used 0,7 ul EcoRI and 0,5 ul PstI to each reaction. Incubated in 37 C for 1h 45 min.

Ligation:
Used Thermo Fisher T4 ligase. Followed the protocol from:
https://tools.thermofisher.com/content/sfs/manuals/MAN0011906_DNAsert_Ligation_Vector_DNA_UG.pdf

Insert DNA molar ratio over backbone: used 1:1

Calculations for 1:1 insert/vector molar ratio
Backbone (pSB1C3): 2070 bp, restricted concentration: 10 ng/ul, 50 ng needed for the reaction -> **5 ul** needed = 39.09 fmol
CAR whole 4928 bp, restricted concentration: 10 ng/ul, 39.09 fmol = 119.0 ng -> **11,9 ul** /restriction reaction needed.

Ligation reaction mix:
5 ul restricted AH009
11,9 ul restricted CAR (Did an own mix for all the four restricted CARs)
0,2 ul T4 ligase
2 ul buffer
0,9 ul water
total 20 ul

Incubated ligations in 23 C for 10 min. Inactivated incubating in 70 C for 5 min. Used 5 ul for transformation.

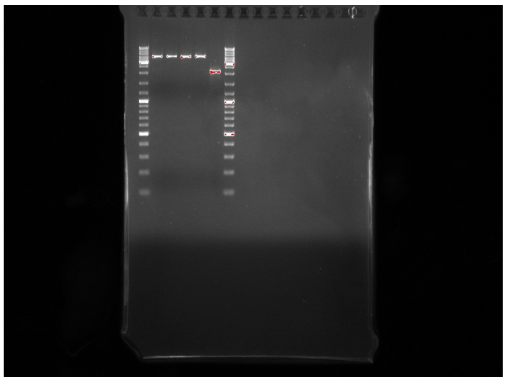
Transformation:
Transformed CAR Gibson 2, CAR Gibson 3, CAR Gibson 4 and CAR Gibson 7 (in pSB1C3 backbone) to TOP10. Followed the protocol. Incubated on ice for 25 min and in 37 C for 40 min before plating.
Plated 50 ul and 200 ul of every reaction on chloramphenicol plates (8 plates in total)

Made a 1,3 % agarose gel with ETBR. Ran 5 ul samples of restrictions with 1 ul LD. Used GeneRuler ladder. Ran the gel for 40 min, 110V

Pipetting order

1. Ladder
2. CAR Gibson 2
3. CAR Gibson 3
4. CAR Gibson 4
5. CAR Gibson 7
6. AH009 (pSB6C1)
7. Ladder

Geldoc_2015-08-17_18hr_25min_CAR_whole_and_AH009_restriction.jpg



According to the gel pic all the restrictions were succesful.

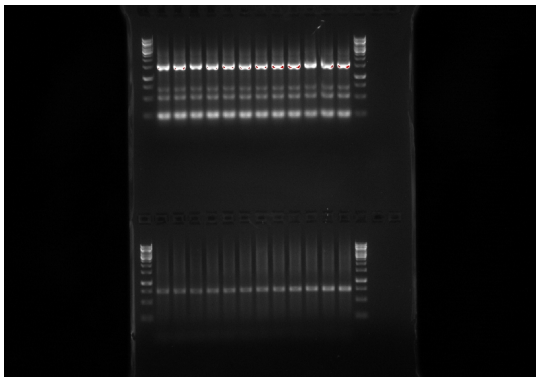
Did restrictions of GFP (with pSB1C3) with EcoRI & XbaI and AH013 and AH015 with EcoRI & SpeI following mostly the protocol. However, the incubation was 1,5 h.

Did ligations of restricted GFP with restricted AH013/AH015 following the T4 ligase protocol. Used 5 ul GFP and 10 ul AH013/AH015 in the ligations.

Transformed the ligations to Top10 CHEM competent cells following the protocol. Plated two 50 ul and 150 ul to CAM+IPTG plates and incubated in 37 C o/n.

Made a 1,3 % agarose gel with EtBr. Run CAR amph part 3 & CAR amph part 4 PCR products in the gel for 25 min with 120 V.
Pipeting order was:
I 1. ladder 2 ul 2.-13. CAR amph part 3 gradient 6 ul 14. ladder 2 ul
II 1. ladder 2 ul 2.-13. CAR amph part 4 gradient 6 ul 14. ladder 2 ul

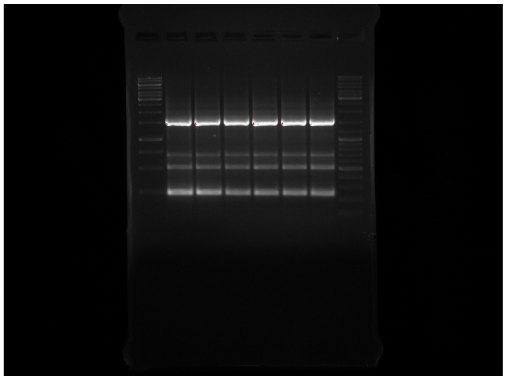
Geldoc_2015-08-17_15hr_37min_Caramphpart34.jpg



Results: PCR reactions of Car amph part 3 & 4 were successful, and PCR products of Car amph part 4 were strored in +4 C. Continued work with Car amph part 3.

Made a 1,3 % agarose gel with EtBr. Run CAR amph part 3 PCR products (6 tubes) for gel purification in the gel for 45 min with 100 V. Pipeting order was:
1. O' GeneRuler ladder 2 ul 2.-3. CAR amph part 3 with 6x Orange LD 18 ul 4.-7. CAR amph part 3 with 6x GeneRuler LD 18 ul 8. GeneRuler Mix ladder 2 ul

Geldoc_2015-08-17_18hr_00min_CAramphpart3.jpg



Did gel purification of CAR amph part 3 following the kit protocol. Added 862,8 ul binding buffer.

NanoDrop result of the purification:

Table2

Sample	DNA (ng/ul)	A260/A280
CAR amph part 3	57,3	1,82

Stored in - 20 C.

18.8.2015

TUESDAY, 8/18

Petra

Note: The backbone used for AtoB & CenA reactions so far has always been pSB1C3 (AH009). Former notes about the backbone may include typos like pSB6C3. The backbone meant is always pSB1C3)

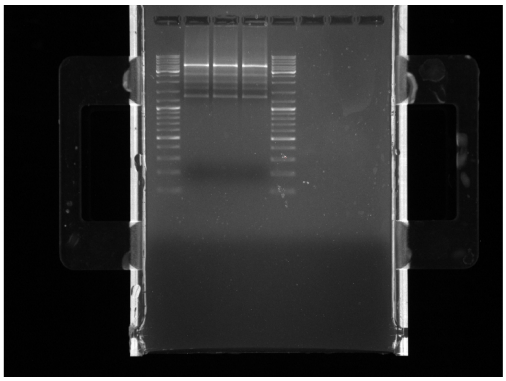
Transfering CAR construct to biobrick backbone (pSB1C3): Checking yesterday's transformations:

- Colonies on every plate (CAR Gibson 2,3,4, 7: 50 ul and 200 ul)
- Colonies not red, so they might contain the right insert
- Decided to do o/n cultures to miniprep and check on gel tomorrow

Purifying CenA-cex-BglX oe-PCR reaction from gel:
Made a 1,3 %agarose gel with ETBR. Ran 20 ul samples of yeaterday's CenA oe-PCR reactions 1-3 on gel with 4 ul LD. Used GeneRuler ladder. Ran the gel for 45 min, 110 V.
Pipetting order:

1. Ladder
2. CenA oe-PCR 1
3. CenA oe-PCR 2
4. CenA oe-PCR 3
5. Ladder

Geldoc_2015-08-18_11hr_31min_CenA_oe-pcr_gel_purification.jpg



Purified the highest bands from the gel.

Purified CenA oe-PCR reactions from gel using GeneJET Gel Extraction kit. Followed the protocol. Final elution was made with 25 ul elution buffer.

CenA oe-PCR gel purification: Nanodrop results

- 3,9 ng/ul
- A260/A280: 1,47
- Result: Purification was unsuccesful, can't be used for Gibson assembly

Purified CenA oe-PCR reaction 4 (15 ul left) with GeneJET PCR purification kit because gel extraction done before didn't work. Followed the protocol. Final elution was made with 25 ul elution buffer.
Nanodrop results:

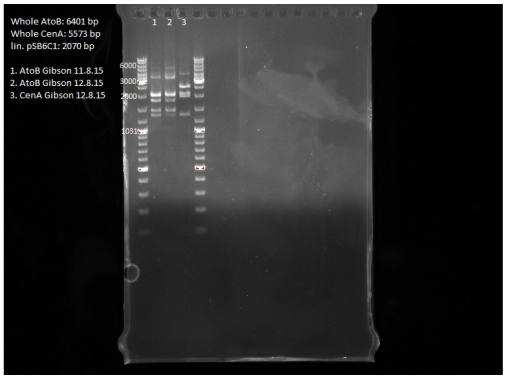
- 11,0 ng/ul
- A260/A280: 1,81
- Decided to try out Gibson with this

Checking former Gibson assembly reactions on gel:
Made a 1,3 % agarose gel with ETBR. Ran all the Gibson assembly reactions made for AtoB and CenA constructs on gel:

- AtoB Gibson 11.8.15: 5 ul reaction, 1 ul LD
- AtoB Gibson 12.8.15: 3 ul reaction, 0,6 ul LD
- CenA gibson 12.8.15: 5 ul reaction, 1 ul LD

De-activated the samples by incubating them in 80 C for 5 min. Added LD after de-activation.
Pipetting order was the same as described before. Used GeneRuler ladder. Ran the gel for 50 min, 110 V.

EDITED_AtoB_CenA_gibson_Geldoc_2015-08-18_12hr_59min.jpg



According to gel picture Gibson reaction mix has worked. Decided to transform the rest of the Gibson reactions again and hope that some of the bands are supercoiled whole plasmids.

Gibson assebmly for whole CenA-cex-BglX
Total amount of DNA in the reaction mix: 0,03 pmol
Vector:insert molar ratio 1:2

Table1

Construct	Lenght	Concentration (ng/ul)	DNA (ng) needed for 0.02 pmol CenA & 0,01 pmol vector	DNA (ul) needed for 0.02 pmol CenA & 0,01 pmol vector
CenaA whole	5573	11	68,88	6,26
pSB1C3 (AH009)	2070	25	12,79	0,51
Total:				6,77
Buffer:				6,77

Pipetted Whole CenA from oe-PCR and linearized pSB1C3 (AH009) backbone to 1,5 ul microcentrifuge tube according to Table 1. Added 6,77 ul 2X Mastermix. Didn't add any water. Incubated in 50 C for 15 min.

Transformation of Gibson reactions:
Transformed 4 Gibson reactions (construct, date when the reaction was made, amount used for transformation):

- CenA 18.8 (made from oe-PCR reaction): 3 ul
- CenA 12.8: 5 ul
- AtoB 11.8:5 ul
- AtoB 12.8: 3,5 ul

Transformed CenA 18.8 (made from oe-PCR reaction) to NEB 5-alpha Competent *E. coli* cells and the others to TOP10. Followed the transformation protocol. Plated 250 ul of each reaction on cholamphenicol plates. Incubated in 37 C overnight.

Making NEB 5-alpha Competent *E. coli* cell stock:
Plated NEB 5-alpha Competent *E. coli* cells on a fresh LB plate (no antibiotics) in the laminar. Incubated in 37 C overnight.

Making o/n cultures of CAR construct with pSB1C3 backbone:
Made 8 o/n cultures

- chose 1 colony per plate (8 plates in total: CAR Gibson 2,3,4, 7: 50 ul and 200 ul)
- Naming: CAR Gibson 2 #1, CAR Gibson 2 #2 ... CAR Gibson 7 #2
- 2 ml LB
- 2 ul chloramphenicol stock (34 ug/ul) per tube
- Plated all the colonies on a fresh CAM plate and incubated the plate and the o/n cultures in 37 C overnight

Yesterday's transformation of AH013/AH015+GFP was successful (there were white colonies), but we don't know which colonies are right because the backbone with the old insert had same antibiotic resistance. Did transformation again using a different backbone (from pSB1C3; 3A method).

Did tetracycline (Tc) LB plates. Pipeted 500 ul Tc (Tc concentration: 10 mg/ml) to 500 g LB agar.

Did restrictions of GFP (with pSB1C3) with PstI & XbaI and pSB1T3 with EcoRI & PstI following mostly the protocol. However, the incubation was 1,5 h.

Did two ligations of restricted GFP and pSB1T3 with restricted AH013/AH015 following the T4 ligase protocol.

In the first ligations used 0,52 ul AH013/AH015, 6,5 ul GFP and 7 ul pSB1T3. In the second ligations used 2 ul AH013/AH015, 2 ul GFP and 2 ul pSB1T3.

Transformed the ligations to Top10 CHEM competent cells following the protocol. The incubation was 50 min. Plated 50 ul and 150 ul to Tc plates and incubated in 37 C o/n.

Did PCR purification of Car amph part 4 following the kit protocol.
NanoDrop result:

Table2

Sample	DNA (ng/ul)	A260/A280
Car amph part 4	63,9	

19.8.2015

WEDNESDAY, 8/19

Petra

Making of chloramphenicol plates
Made more choramhenicol plates. Followed the protocol. Stored the plates to +7 C fridge.

Miniprepping CAR constucts in pSB1C3 backbone
Miniprepped 8 o/n cultures of CAR2 #1 ... CAR7 #2 using NucleoSpin plasmid EasyPure Miniprep kit. Nanodrop results are in Table 1.

Table1

Construct	Concentration (ng/ul)	A260/A280
CAR2 #1 in pSB1C3	38,8	1,94
CAR2 #2 in pSB1C3	37,4	1,93
CAR3 #1 in pSB1C3	33,7	2,01
CAR3 #2 in pSB1C3	33,6	1,95
CAR4 #1 in pSB1C3	34,8	1,99
CAR4 #2 in pSB1C3	34,8	1,96
CAR7 #2 in pSB1C3	31,8	1,98
CAR2 #1 in pSB1C3	47,6	1,87

Restricting miniprepped CAR + pSB1C3 -constructs
Restricted 250 ng samples of each miniprepped plasmid to check on gel whether the plasmids contain right-sized insert. Used NeBuffer 3.1 for restriction with EcoRI & Pst1.
Restricted the constructs (CAR2 #1 ... CAR7 #2) with EcoRI and PstI according to the protocol. Amounts of DNA and water used are listed in Table 1.

Table2

Construct	DNA pipetted for 250 ng (ul)	Water added (ul)
CAR2 #1 in pSB1C3	6,44	14,86
CAR2 #2 in pSB1C3	6,68	14,62
CAR3 #1 in pSB1C3	7,42	13,88
CAR3 #2 in pSB1C3	7,44	13,86
CAR4 #1 in pSB1C3	7,18	14,12
CAR4 #2 in pSB1C3	7,18	14,12
CAR7 #2 in pSB1C3	7,86	13,44
CAR2 #1 in pSB1C3	5,26	16,04

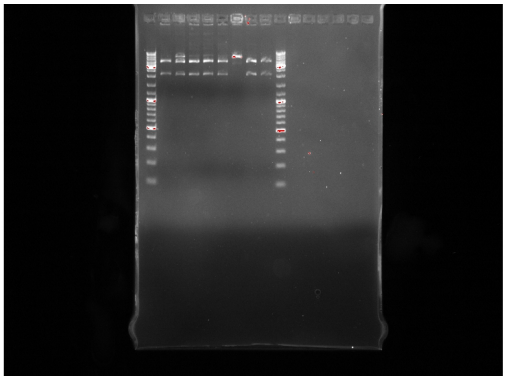
Used 0,7 ul EcoRI and 0,5 ul PstI to each reaction. Incubated in 37 C for 1h.

Checking CAR Gibson restrictions on gel:
Checked on gel which colonies contain the right insert. Made a 1,3 % agarose gel with ETBR. Ran 5 ul samples of each restriction reaction with 1 ul LD on gel. Used GeneRuler ladder. Ran the gel for 40 min, 120 V.
Pipetting order:

1. Ladder
2. CAR 2 #1
3. CAR 2 #2
4. CAR 3 #1
5. CAR 3 #2
6. CAR 4 #1
7. CAR 4 #2
8. CAR 7 #1
9. CAR 7 #2
10. Ladder

Whole CAR: 4928 bp

Geldoc_2015-08-19_16hr_42min_car_in_psb1c3_restricrion.jpg



According to the gel picture 7 samples contain right-sized insert: CAR 2 #1, CAR 2 #2, CAR 3 #1, CAR 3 #2, CAR 4 #1, CAR 7 #1, CAR 7 #2

Checking yesterday's transformations:
Checked Gibson assembly transformations:

- AtoB Gibson 11.8: 2 red colonies, 1 colorless colony
- AtoB Gibson 12.8: several red colonies, 3 colorless colonies
- CenA Gibson 12.8: 1 red colony, 4 colorless colonies
- CenA Gibson 18.8: No colonies, discarded the plate

Chose all the colorless colonies to make o/n cultures.

- 2 ul LB
- 2 ul chloramphenicol stock (34 mg/ml)
- 8 cultures in total
- Naming: AtoB Gibson 11.8 #1 (construct name, method (Gibson), date when Gibson was made, colony number)

Plated all the colonies on a fresh cam plate at the same time. Incubated in 37 C with shaking overnight.

Yesterday's transformation of AH013/AH015+GFP+pSB1T3 was unsuccessful (no colonies in the plates).

Did restrictions of GFP (with pSB1C3) from the miniprepared plasmids of colony 3 colony 4 with PstI & XbaI and pSB1K3 (AH008) with EcoRI & PstI following mostly the protocol. However, the incubation was 1,5 h. Stored the restrictions in - 20 C.

Diluted BBa_K608003 (strong promoter, medium rbs; AH045) from the plate 1 (5A).

Transformed AH045 to Top10 CHEM competent cells. Plated 50 ul and 150 ul to CAM plates. Incubated the plates o/n.

20.8.2015

THURSDAY, 8/20

Petra

Miniprepping yesterday's AtoB and CenA Gibson o/n cultures:
CenA Gibson colony #4 culture had turned red, so discarded it. Miniprepped CenA Gibson #1 - #3 and AtoB Gibson #1 - #4 using NucleoSpin Plasmid EasyPure kit. Nanordop results are in Table 1.

Table1

Costruct & colony number	Concentration (ng/ul)	A260/A280
CenA Gibson #1	45,3	1,88
CenA Gibson #2	36,4	1,86
CenA Gibson #3	37,1	1,85
AtoB Gibson #1	37,4	1,86
AtoB Gibson #2	31,9	1,88
AtoB Gibson #3	47,7	1,75
AtoB Gibson #4	39,1	1,91

Checking which AtoB and CenA constructs contain right-sized insert:
Restricted 250 ng samples of AtoB and CenA minipreps according to the restriction protocol. Restricted all the samples with EcoRI and PstI. Used 2,5 ul NeBuffer 3.1, 0,7 ul EcoRI and 0,5 ul PstI. Amounts of DNA and water used are listed in Table 2:

Table2

Costruct & colony number	DNA (ul) pipetted for 250 ng	Wated (ul) added
CenA Gibson #1	5,52	15,78
CenA Gibson #2	6,88	14,42
CenA Gibson #3	6,74	14,56
AtoB Gibson #1	6,68	14,62
AtoB Gibson #2	7,84	13,46
AtoB Gibson #3	5,24	16,06
AtoB Gibson #4	6,40	14,90

Total volume of restriction reactions: 25 ul
Incubated the samples in 37 C for 1 h and inactivated in 80 C for 20 min.

Made a 1,3 % agarose gel with ETBR. Ran 5 ul samples of the restrictions on gel with 1 ul LD. Used GeneRuler ladder. Ran the gel for 50 min, 110 V.
Pipetting order:

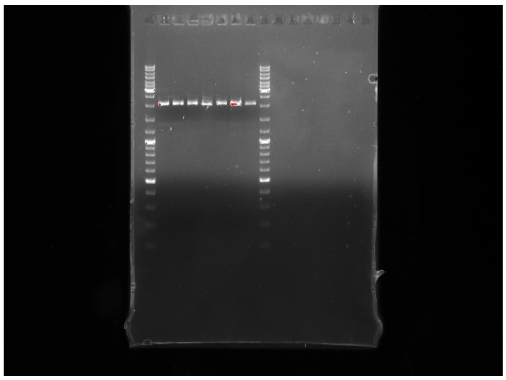
1. Ladder
2. AtoB Gibson #1

3. AtoB Gibson #2
4. AtoB Gibson #3
5. AtoB Gibson #4
6. CenA Gibson #1
7. CenA Gibson #2
8. CenA Gibson #3
9. Ladder

Construct sizes:

- whole AtoB: 6401 bp
- whole CenA: 5573 bp
- pSB1C3 backbone: 2070

Geldoc_2015-08-20_14hr_50min_Atob_cena_gibson.jpg



According to the gel picture none of the colonies contain the right insert. Discarded all the samples.

Analysing CAR (in AH043 backbone) sequencing results:
Sequencing of CAR colony 2, CAR colony 3, CAR colony 4 and CAR colony 5 (from Gibson assembly) was unsuccesful. Only primer P011 annealed to the template and produced some data for analysing. It is possible that none of the samples contain the right insert but it's hard to say.

Sequencing CAR (in pSB1C3):
Decided to sequence 3 samples of whole CAR in pSB1C3 backbone even that CAR in AH043 sequencing results were not good. Chose the samples that looked the best and right-sized in the gel picture from yesterday:

- CAR2 #1
- CAR3 #2
- CAR4 #1

Used primers VF2, P075, P077, P079, P081, P083, P085 and VR. Made the seguencing samples according to FIMM's instructions.

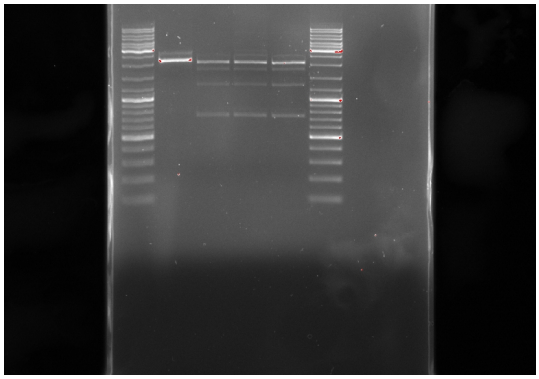
After making the sequencing samples all the miniprepped DNA of CAR2 #1, CAR3 #2 and CAR4 #1 was finished. To miniprep more tomorrow made 1 o/n culture of each:

- 2 ml LB
- 2 ul chloramphenicol stock (34 mg/ml)

Incubated the cultures in 37 C with shaking overnight.

Made a 1,3 % agarose gel with EtBr. Run the restrictions of 19.8. pSB1K3, 18.8. GFP c3, 19.8. GFP c3 and 19.8. GFP c4 in the gel for 45 min with 100 V. It seems that all the bands contained right sized pieces.

Geldoc_2015-08-20_13hr_03min_pSB1K3_GFPold_newc3c4.jpg



Did o/n cultures of AH045, GFP (with pSB1C3) c2, c3, c4, c5 & c6 in 2 ml LB with 2 ul CAM.

21.8.2015

FRIDAY, 8/21

Petra

Miniprepped yesterday's o/n cultures (whole CAR in pSB1C3 backbone) using NucleoSpin Plasmid EasyPure kit. Followed the protocol. Nanodrop results are in Table 1.

Table1

Construct	Concentration (ng/ul)	A260/A280
CAR2 #1 in pSB1C3	48,2	1,91
CAR3 #2 in pSB1C3	79,0	2,03
CAR4 #1 in pSB1C3	28,5	1,92

Transforming CAR in AH043 and CAR in pSB1C3 backbone to Pauli Kallio's BL21(DE3) (=DEL1) cells:
Decided to transform CAR constructs in AH043 and pSB1C3 backbones to DE3 cells to test CAR construct with SDS-PAGE next week.
Transformed 7 samples in total:

1. CAR2 in AH043
2. CAR3 in AH043
3. CAR4 in AH043
4. CAR7 in AH043
5. CAR2 #1 in pSB1C3
6. CAR3 #2 in pSB1C3
7. CAR4 #1 in pSB1C3

Used so many samples because CAR in AH043 backbone sequencing samples didn't give us any data to say which colonies contain the right insert. CAR in pSB1C3 samples have been sent to sequencing but we don't have the results yet.
Followed the transformation protocol. Used 2 ul DNA for every transformation.
Plated samples 1-4 (CAR in AH043) on ampicillin plates and samples 5-7 on cloramphenicol plates. Plated every sample on 2 plates: 50 ul in one and 200 ul in the other. Stored the plates in room temperature with no light over the weekend.

Did minipreps of yesterday's o/n cultures of AH045, GFP c2, c3, c4, c5 & c6 following mostly the kit protocol. However, plasmids were eluted with 20 ul elution buffer.

NanoDrop results:

Table2

Sample	DNA (ng/ul)	A260/A280
GFP c2	98,3	1,87
GFP c3	727,3	1,83
GFP c4	136,6	1,85
GFP c5	102,8	1,88
GFP c6	111,5	1,88
AH045	57,2	1,85